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α-Glucuronidase and Other Hemicellulase Activities of *Fibrobacter succinogenes* S85 Grown on Crystalline Cellulose or Ball-Milled Barley Straw

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Fibrobacter succinogenes **produces an α-glucuronidase which cleaves 4-*O*-methyl-α-D-glucuronic acid from birch wood 4-*O*-methyl-α-D-glucuronoxylan. Very low levels of α-glucuronidase activity were detected in extracellular enzyme preparations of *F. succinogenes* on birch wood xylan substrate. The release of 4-*O*-methyl-α-D-glucuronic acid was enhanced when the birch wood xylan substrate was predigested by either a purified *Schizophyllum commune* xylanase or a cloned *F. succinogenes* S85 xylanase. These data suggest that the α-glucuronidase is unable to cleave 4-*O*-methyl-α-D-glucuronic acid from intact xylan but can act on unique low-molecular-weight glucuronoxylan fragments created by the cloned *F. succinogenes* xylanase. The cloned xylanase presumably must account for a small proportion of the indigenous xylanase activity of *F. succinogenes* cultures, since this xylanase source does not support high glucuronidase activity. The α-glucuronidase and associated hemicellulolytic enzymes exhibited higher activities in culture fluid from cells grown on ball-milled barley straw than in that of cellulose-grown cells. The profile of xylanases separated by isoelectric focusing (zymogram) of culture filtrate from cells grown on barley straw was more complex than that of culture filtrates from cells grown on cellulose. These data demonstrate that *F. succinogenes* produces an α-glucuronidase with an exacting substrate specificity which enables extensive cleavage of glucuronic acid residues from xylan as a consequence of synergistic xylanase action.**

The hemicellulose contents of grasses, legumes, and cereal straw range between 21 and 37% depending on the maturity and plant structure analyzed (41). A major polymer in hemicellulose is the arabinoglucuronoxylan with 4-*O*-methyl-α-D-glucuronyl residues linked (1→2) to xylose residues (41). The glucuronic acid concentration of forages and cereal straws varies from 6 to 11% (wt/wt) in *Trifolium protense* (red clover) (2) and from 2 to 5% (wt/wt) in cereal straw and grasses (3, 5, 40), and it increases with maturity (40). The molar ratio of, for example, xylose to uronic acid in oat leaves and stems was 2.2 to 4.4:1 (10). In oat plants, both glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid are present, and the extent of 4-*O*-methylation increases with maturity. In birch wood xylan, the 4-*O*-methyl-α-D-glucuronic acid residues are irregularly distributed on the xylan backbone (32). The structural organization of other glucuronoxylans has not been characterized. There is indirect evidence which suggests that the carboxyl groups of 4-*O*-methyl-α-D-glucuronic acid residues are ester linked to lignin hydroxyl groups, thereby providing cross-links between lignin and cell walls (11, 13, 27) analogous to the phenolic acid bridges reported by Iiyama et al. (23) and Scalbert et al. (35). Since 4-*O*-methyl-α-D-glucuronic acid residues are prominent constituents of hemicellulose, they presumably decrease the accessibility of microbial xylanases to xylan, thereby reducing forage digestibility. From studies on the biodegradation of plant cell wall polymers by ruminal, microorganisms, we know that the predominant fibrolytic bacterium *Fibrobacter succinogenes* and fungi possess cellulases, xylanases, acetyl xylan esterases, ferulic acid esterases, and arabinofuranosidases (7, 17, 18, 28, 29).

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In this study, we tested for the presence of α-glucuronidase and determined the activities of related fibrolytic enzymes produced by *F. succinogenes* when grown on crystalline cellulose and on ball-milled barley straw (BMBS). These substrates were chosen because most fibrolytic enzymes appear to be produced when *F. succinogenes* is grown on cellulose (17), while barley straw provides a recalcitrant complex substrate which contains 4-*O*-methyl-α-D-glucuronic acid covalently linked to the xylan backbone (42).

MATERIALS AND METHODS

Bacteria and culture conditions. The bacteria used in this study were *F. succinogenes* S85 (20) and *Escherichia coli* X14 (*E. coli* HB101 harboring pBR322 with a *Bam*HI insert from *F. succinogenes* S85 which codes for a xylanase [unpublished data]). The X14 xylanase gene lacks homology with the BX1 xylanase cloned by Sipat et al. (38). *F. succinogenes* was grown in the chemically defined medium of Scott and Dehority (37) with either Avicel microcrystalline cellulose PH105 (FMC Corp., Marcus Hook, Pa.) or BMBS at 0.5% (wt/vol) as the sole source of carbohydrate. Bacterial biomass was estimated by the biuret method for total protein (21). The cell-free culture supernatant from *F. succinogenes* cultures grown on cellulose or BMBS was recovered after 72 and 144 h, respectively (protein concentrations of the cultures reached approximately 1.0 mg ml^{-1}), and was concentrated 10-fold by ultrafiltration through a membrane (PM-10; Amicon Corp., Lexington, Mass.). The enzyme was diluted 10-fold with 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 6.5) and reconcentrated. This fraction was used because in the past it has been found to contain the major hydrolytic enzymes of *F. succinogenes* (17, 36). The *E. coli* X14 clone was grown in LB

medium (34) containing 50 μg of ampicillin ml^{-1}. The ampicillin (sodium salt, 10 mg ml^{-1}) was filter sterilized and added to the precooled sterile LB medium. When the growing culture reached the late exponential phase of growth, cells were harvested by centrifugation at 16,000 × *g* for 15 min at 4°C.

Periplasmic xylanase was recovered from *E. coli* X14 by osmotic shock following the procedure of Cornelis et al. (12) and then concentrated 10-fold by ultrafiltration through a PM-10 membrane diluted 10-fold in 0.1 M MES buffer (pH 6.5) and reconcentrated again. The specific activity of the enzyme fraction was 4.2 μmol/min/mg of protein.

Enzyme assays. Xylanase was assayed by using the water-soluble fraction of oat spelt xylan at a final concentration of 1% (wt/vol) in 0.1 M MES buffer (pH 6.5). The water-soluble fraction of oat spelt xylan was prepared by suspending 4% (wt/vol) of the whole oat spelt xylan in water and stirring for 4 h at 20°C and then centrifuging at 16,000 × *g* for 10 min. The supernatant fluid was saved and freeze-dried. Reducing sugar released in the xylanase assay was quantified with the Nelson-Somogyi reagent as described by Schellhorn and Forsberg (36). Extended xylan hydrolysis was assayed as above, except that whole oat spelt xylan or rye arabinoxylan (4) was used as the substrate. The reaction was terminated after 24 h by steaming (15 min). Products of the reaction were assayed by high-pressure liquid chromatography with a HPX-42A column (Bio-Rad Laboratories), with distilled water as the eluent at a flow rate of 0.6 ml min^{-1}. α-Glucuronidase activity was estimated from the release of 4-*O*-methyl-α-D-glucuronic acid from a 1% (wt/vol) suspension of birch wood xylan in 0.05 M MES buffer (pH 6.5) containing 0.02% (wt/vol) sodium azide at 37°C. In the assays reported in Table 1, birch wood xylan was predigested at a concentration of 1.25% (wt/vol) for 18 h in 0.05 M MES (pH 6.5) by either purified endoxylanase from *Schizophyllum commune* (9) or by a xylanase derived from the periplasmic contents of the xylanase clone *E. coli* X14 (final activity, 1.0 U ml^{-1}). When applicable, predigestion of the birch wood xylan substrate with xylanase was terminated after 18 h by heating at 85°C for 30 min. This treatment completely inactivated both the *S. commune* and the *E. coli* X14 endoxylanases. For assays of glucuronidase activity, the digested birch wood xylan was diluted by 20% (vol/vol) with the same buffer and α-glucuronidase enzyme preparation (containing ≈1.0 U of indigenous xylanase) and incubated for a further 18 h. 4-*O*-Methyl-α-D-glucuronic acid released was determined essentially as described by Fontana et al. (16), which involved trimethylsilyl (TMS) derivatization of reaction products with *N*,*O*-bis(TMS)trifluoroacetamide. In our system, a 2-μl injection of the derivatized products was applied to an OV-1 glass column (2 m long by 2-mm inner diameter) with nitrogen as the carrier gas (flow rate of 40 ml min^{-1}). The injector and oven temperatures were both 180°C, and the detector temperature was 250°C. 4-*O*-Methyl-α-D-glucuronic acid TMS derivatives were also analyzed on a fused silica capillary column (0.32 mm by 15 m) coated with DB-5 (J & W Scientific, Folsom, Calif.) run isothermally at 180°C. 4-*O*-Methyl-α-D-glucuronic acid was identified by reference to a mixed standard composed of 4-*O*-methyl-α-D-glucuronic acid, methylaldiouronic acid, and methyltriouronic acid in a ratio of 4:2:1 (wt/wt/wt) prepared as described by Fontana et al. (16). β-Xylosidase, β-glucosidase, α-L-arabinofuranosidase, and acetyl xylan esterase activities were measured by using *p*-nitrophenol derivatives of β-D-xylopyranoside, β-D-glucopyranoside, α-L-arabinofuranoside, and acetic acid, respectively, in a microtiter plate assay (22).

TABLE 1. Extracellular α-glucuronidase from *F. succinogenes* assayed with and without xylanase pretreatment of birch wood xylan[a]

Treatment	Release of 4-*O*-methyl-α-D-glucuronic acid[b]	
	μmol ml^{-1} ± SEM	%
$S85_{CEL}$	0	
$S85_{BMBS}$	0	
SC	0	
X14	0	
SC[c] + $S85_{CEL}$	0.38 ± 0.05	8.2
SC + $S85_{CEL}$	1.08 ± 0.03	22.4
X14[c] + $S85_{CEL}$	0.42 ± 0.05	8.7
X14 + $S85_{CEL}$	1.24 ± 0.01	25.6
X14[c] + $S85_{BMBS}$	0.58 ± 0.03	11.2
X14 + $S85_{BMBS}$	3.68 ± 0.06	76.5

[a] Abbreviations: $S85_{CEL}$, extracellular enzyme from *F. succinogenes* S85 grown on cellulose; $S85_{BMBS}$, extracellular enzyme from strain S85 grown on BMBS; SC, *S. commune* xylanase; X14, clone X14 periplasmic xylanase.

[b] The approximate concentration of 4-*O*-methyl-α-D-glucuronic acid in birch wood xylan is 10% (43).

[c] After incubation for 18 h with either *S. commune* or X14 xylanase, the preparation was heated to 85°C to inactivate the enzyme prior to addition of extracellular enzyme from *F. succinogenes*. The treatment below was the same, except the reaction mixture was not heated prior to addition of *Fibrobacter* enzyme.

Ferulic acid esterase was measured by the method of McDermid et al. (29). Cinnamic acid esterase was assayed according to the method of Hu et al. (22). Protein was determined by the dye-binding method of Bradford (8), with bovine serum albumin as the standard.

One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol of product per min. Specific activity was defined as units per milligram of protein. The statistical analysis consisted of a one-way analysis of variance and then Tukey's least significant difference test (39).

GC-MS. Gas chromatography-mass spectrum (GC-MS) analysis was performed with a mass spectrometer (model MS890; Kratos, Manchester, United Kingdom). The sample was separated on a 30-m DB-5 fused silica capillary column. The gas flow rate was 1 ml min^{-1}, and a split time of 45 s was used for all analyses. The injection temperature was 250°C, while the column was held at 80°C for 2 min; this was followed by a gradient of 25°C/min to 150°C, and then a gradient of 15°C/min to 280°C and then held for 2 min. The ion source temperature was 220°C, and the mass range analyzed was 44 to 600.

IEF. Isoelectric focusing (IEF) was performed as described in the Bio-Rad Bio-Phoresis Horizontal Electrophoresis Cell Manual (Bio-Rad Chemical Division, Richmond, Calif.) except that 0.5% (wt/vol) of 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) detergent was included in the gel. Zymogram analyses were performed using either the water-soluble fraction of oat spelt xylan or rye flour arabinoxylan as the substrate in an agarose gel overlay following the procedure outlined by Royer and Nakas (33).

RESULTS

α-Glucuronidase of *F. succinogenes* S85. The amounts of 4-*O*-methyl-α-D-glucuronic acid released from birch wood xylan during extended hydrolysis by the extracellular enzymes of *F. succinogenes* S85 grown with either cellulose or

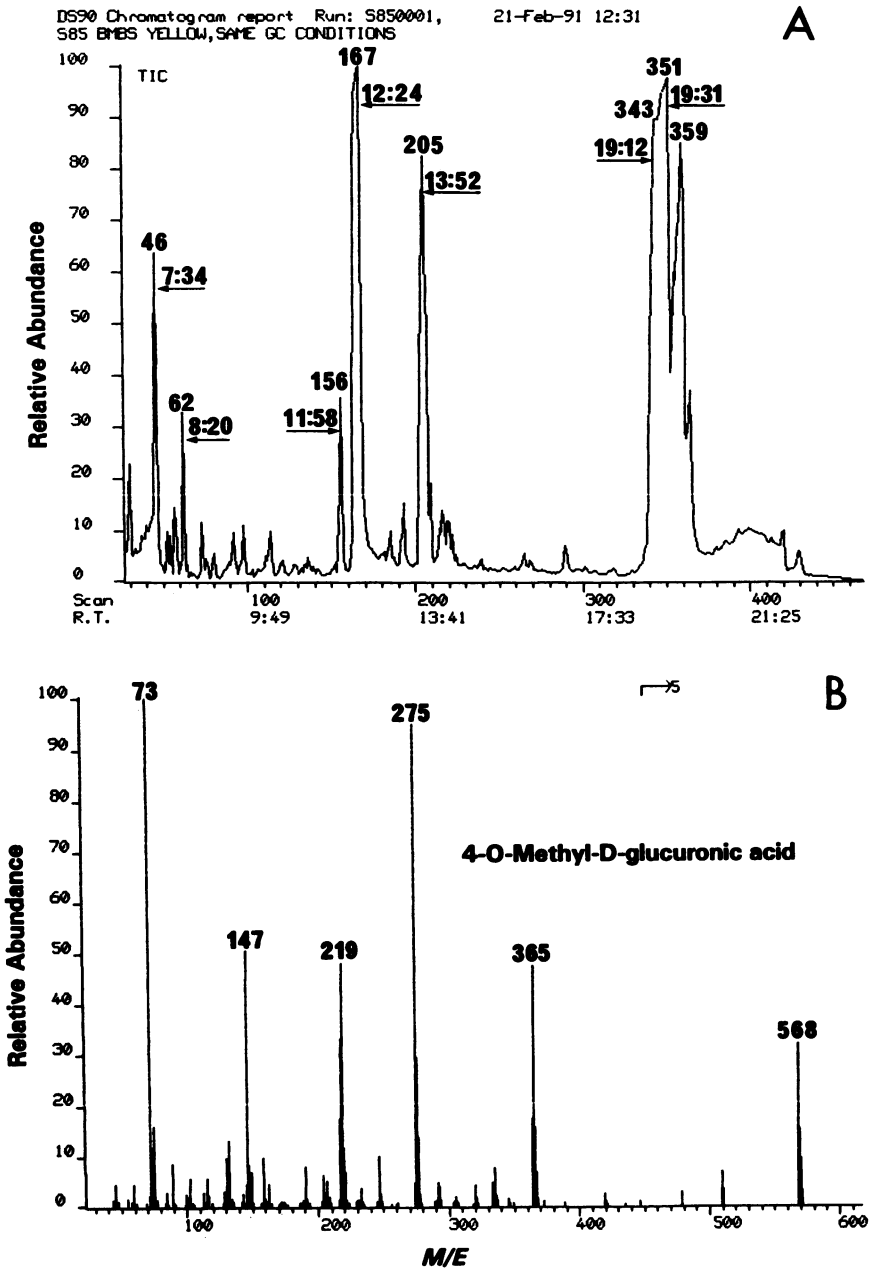

FIG. 1. GC-MS analysis of the TMS-derivatized products from birch wood xylan hydrolysis by the extracellular hemicellulases of *F. succinogenes* grown on BMBS. (A) GC profile of the TMS derivatives of xylan hydrolysis products; (B) MS analysis of the TMS-glucuronic acid peak (scan 205 at 13 min and 52 s).

BMBS as the carbon source are shown in Table 1. Neither extract was able to release glucuronic acid over an 18-h hydrolysis period. In both cases, 4-*O*-methyl-α-D-glucuronic acid release was first detected after 120 h of incubation at 37°C and was maximal after 240 h of incubation, at which time almost 100% of the available 4-*O*-methyl-α-D-glucuronic acid was released. The release of 4-*O*-methyl-α-D-glucuronic acid from birch wood xylan was enhanced by preincubation of the substrate with purified xylanase A from *S. commune*. Further increases in the release of 4-*O*-methyl-α-D-glucuronic acid were seen when the added xylanase was not inactivated prior to incubation with the *F. succinogenes* S85 extracellular enzyme preparation. When the *S. commune* xylanase was substituted with an equal activity of a cloned xylanase (X14) from *F. succinogenes* S85, a greater degree of synergism was noted, as reflected in the larger quantities of 4-*O*-methyl-α-D-glucuronic acid released. The release of 4-*O*-methyl-α-D-glucuronic acid by extracellular enzyme from BMBS-grown *F. succinogenes* cells was substantially higher than that of cellulose-grown cultures. The difference was greatest when the X14 xylanase was not denatured by heat before the addition of the *F. succinogenes* extracellular enzyme. In this case, greater than 77% of the total 4-*O*-methyl-α-D-glucuronic acid was released after 18 h

TABLE 2. Enzymes in the extracellular culture fluid of *F. succinogenes* S85 grown on cellulose and on BMBS[a]

Enzyme	*F. succinogenes* $S85_{CEL}$		*F. succinogenes* $S85_{BMBS}$	
	mU ml^{-1} ± SEM	mU mg^{-1}	mU ml^{-1} ± SEM	mU mg^{-1}
Xylanase	86.7 ± 2.5	677.0	113.4 ± 0.3[b]	1,134
Carboxymethyl cellulase	34.9 ± 1.6	268.2	66.8 ± 0.2[b]	668
Xylobiase	3.7 ± 0.2	28.5	6.4 ± 0.1[b]	64
α-L-Arabinofuranosidase	8.2 ± 0.4	63.1	9.9 ± 0.3	99
Acetyl esterase	8.2 ± 0.3	63.1	21.3 ± 0.2[b]	213
Ferulic acid esterase	0.72 ± 0.09	5.5	0.8 ± 0.07	8
Coumaric acid esterase	0.4 ± 0.08	2.8	0.5 ± 0.04	5

[a] Water-soluble fraction of oat spelt xylan used as substrate for xylanase assays.
[b] Significantly different ($P \leq 0.05$) from the enzymatic activities of cultures grown on cellulose.

compared with 26% released by a similar incubation with extracellular enzyme from a cellulose-grown *Fibrobacter* culture. Neither extracellular enzyme from *F. succinogenes* was able to release 4-*O*-methyl-α-D-glucuronic acid from aldobiouronic acid [2-*O*-(4-*O*-methyl-α-D-glucuronic acid)-D-xylose].

GC-MS analysis of hydrolysis products. When the products of hydrolysis of birch wood glucuronoxylan were analyzed by GC-MS, the major monomeric products were D-xylose at 12 min and 24 s and 4-*O*-methyl-α-D-glucuronic acid at 13 min and 52 s (Fig. 1A). The components eluted at 19 min appeared to be high-molecular-weight materials. The molecular weight of the TMS-xylose parent compound was 525. The TMS-xylose derivatives had major signals at *m/e* of 525, 510, 307, 217, 147, and 103 which corresponded to the species expected from xylose (data not shown).

TMS 4-*O*-methyl-α-D-glucuronic acid has a molecular weight of 583. The observed fragmentation pattern of this compound consisted of major signals at *m/e* 568, 365, 275, 219, and 147, which were identical to a TMS-4-*O*-methyl-α-D-glucuronic acid standard analyzed at the same time. The absence of a signal at *m/e* 583 for the parent compound is due to the lability of the 4-*O*-methyl group at the high temperature of the injector (26).

Hemicellulases produced by *F. succinogenes* S85 grown on either cellulose or BMBS. The major extracellular fibrolytic enzymes produced by *F. succinogenes* grown with either BMBS or cellulose as the carbon source are presented in Table 2. The representative xylan-degrading enzymes were found in both preparations. However, in comparison with cultures grown on cellulose, the cell-free culture fluid of cells grown on BMBS contained almost double the amount of endo-1,4-β-xylanase, endoglucanase, xylobiase, and acetyl esterase.

Zymogram analysis of xylanases. The extracellular enzyme preparations from *F. succinogenes* S85 grown on either cellulose or BMBS were analyzed by IEF between pH 3 and 10. Enzyme patterns were analyzed by zymograms using either oat spelt xylan or rye arabinoxylan as the substrate. Only the zymogram with rye arabinoxylan as the overlay substrate is shown. Similar profiles were observed with both xylan substrates, but the banding was more pronounced with arbinoxylan (Fig. 2). Both extracts appeared to contain as many as nine xylanase activity bands. However, only three bands with pI values of 6.2, 6.7, and 7.1 were common to both extracts (Fig. 2, arrowheads), thereby suggesting that different enzymes were expressed by BMBS-grown cells.

Hydrolysis products of oat spelt xylan and rye arabinoxylans. Table 3 shows the hydrolysis products produced during an 18-h incubation of either oat spelt xylan or rye flour arabinoxylan with extracellular enzyme preparations from *F. succinogenes* S85 grown with either cellulose or BMBS as the carbon source. The enzyme produced during growth on BMBS hydrolyzed oat spelt xylan more efficiently than an equivalent quantity of enzyme produced with cellulose as the carbon source (22% versus 40% total carbohydrate in xylose equivalents). There was no significant difference in the ability of either enzyme preparation to degrade rye arabinoxylan. Both extracellular enzyme preparations were able to hydrolyze greater than 95% of the available carbohydrate to glucose, arabinose, and xylooligosaccharides with chain lengths less than or equal to that of xylotetraose. Unlike the hydrolysis of oat spelt xylan, no xylose was released from the rye flour arabinoxylan.

DISCUSSION

F. succinogenes S85 has been shown to possess α-glucuronidase. This is the first report of this enzyme in a ruminal organism, although it is present in a number of fungi and bacteria from other environments. They include the fungi *Trichoderma reesei* (15), *Agaricus bisporus* (31), *Pleurotus ostreatus* (31), *Aspergillus niger* (24), *S. commune* (24), and

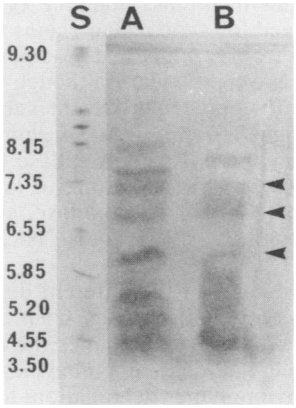

FIG. 2. IEF and zymogram analysis of the extracellular xylanase enzymes from *F. succinogenes* grown on either cellulose or BMBS as the carbon source. Lanes: S, IEF standards stained with Coomassie blue; A, cellulose-grown cells; B, BMBS-grown cells. A total of 0.005 U of xylanase was added to each lane for the zymogram analysis. Arrows on the right indicate proteins in the two lanes that have similar pIs. Numbers on the left are the pIs of protein standards.

TABLE 3. Mono- and oligosaccharides arising from the hydrolysis of either oat spelt xylan or rye arabinoxylan by xylanase enzymes in the cell-free extracellular culture fluid of *F. succinogenes* grown on either cellulose or BMBS as the carbon source

Assay substrate	Growth substrate	Composition of hydrolysis products (%, wt/wt)						
		Xylose	Xylobiose	Xylotriose	Xylotetraose	Arabinose	Glucose	Total
Oat spelt xylan	Cellulose	3.0	9.2	8.1	2.0	2.0	0.0	22.4
	BMBS	5.9	17.6	11.4	2.5	2.7	0.0	40.1[a]
Rye arabinoxylan	Cellulose	0.0	15.6	21.6	34.0	17.4	8.1	96.5[b]
	BMBS	0.0	23.1	23.1	26.4	21.1	9.7	103.4[b]

[a] Significantly different ($P \leq 0.05$) from the total oligosaccharide concentration produced by cells grown on cellulose.
[b] Not significantly different ($P \geq 0.05$) from each other, but significantly different ($P \leq 0.05$) from the concentration of products arising from the hydrolysis of oat spelt xylan.

Thermoascus aurantiacus (25) and the bacteria *Streptomyces flavogriseus* and *Streptomyces olivochromogenes* (24).

The α-glucuronidase from *F. succinogenes* was not reproducibly detected in extracellular enzyme preparations with birch wood xylan as the substrate but was readily assayed with substrate predigested with either a purified xylanase from *S. commune* or a cloned xylanase from *F. succinogenes* expressed in *E. coli*. The *S. commune* xylanase A cleaves the unsubstituted xylan backbone to xylose and xylobiose (30), while the *E. coli* X14 enzyme cleaves oat spelt xylan to xylose, xylobiose, and xylotetraose (unpublished data), but the number of xylose residues in the xylan chains of the glucuronoxylan fragments may be different. A requirement for a low-molecular-weight glucuronoxylan substrate was previously reported for the glucuronidases from *A. bisporus* (31) and *S. olivochromogenes* (24). However, the *A. bisporus* and *P. ostreatus* α-glucuronidases were shown to cleave 2-*O*-(4-*O*-methyl-α-D-glucuronic acid)-D-xylobiose. Since *F. succinogenes* does not cleave this substrate, a higher oligosaccharide chain length may be necessary. The increased extent of degradation of predigested xylan in the presence of active xylanase in conjunction with the α-glucuronidase suggests that cleavage of 4-*O*-methyl-α-D-glucuronyl residues makes available cleavage sites for the cooperative action of the xylanase. This is consistent with the finding of Rosell and Svensson (32) that the 4-*O*-methyl-α-D-glucuronic acid residues are irregularly distributed, or perhaps distributed in clumps, along the xylan backbone. In contrast to the need for short xylan chains for cleavage, the α-glucuronidases from *A. niger* and *S. commune* were active on high-molecular-weight xylan, although the activity was even greater on low-molecular-weight substrate (24). The thermophilic fungus *Thermoascus auranticus* appears to produce a similar type of α-glucuronidase (25). It exhibited 55% of the hydrolytic activity on native larch xylan that it had on 4-*O*-methyl-α-D-glucuronoxylose, and it had similar activities on substrates with a xylose backbone of one to seven residues.

These reports document the different degrees of synergy that occur between glucuronidases from different sources with a xylanase. It further exemplifies the need for cooperative action of cell wall-degrading enzymes to enable more rapid and extensive polymer degradation, as had previously been demonstrated for action of xylanases with acetyl xylan esterase (6) and with α-L-arabinofuranosidase (19).

Growth of *F. succinogenes* on BMBS resulted in increased activities of most plant cell wall degradation enzymes tested and in a different IEF zymogram profile of xylanases. The xylanolytic activity of extracellular enzymes from BMBS-grown cells had a substantially greater capacity to degrade oat spelt xylan, while both sources of enzyme cleaved arabinoxylan to a similar extent. This could be due to increased α-glucuronidase activity, but we cannot preclude the influence that a change in the distribution of xylanases may have (Fig. 2). *F. succinogenes* possesses at least four different xylanases as documented as by the purification of unique xylanases and by gene cloning (unpublished data). It is very obvious that the major *F. succinogenes* xylanase(s) has a substrate specificity more suited to cleave arabinoxylan. This would suggest that the X14 xylanase is a minor component of the xylanases synthesized by *F. succinogenes*. Perhaps the X14 xylanase is produced to a greater extent when cells are grown on BMBS.

The synthesis of α-glucuronidase in *F. succinogenes* adds another important enzyme to the broad range of hydrolytic activities produced by the bacterium. Because of the ubiquitous presence of a 4-*O*-methyl-α-D-glucuronic acid in hemicelluloses of grasses and legumes, the glucuronidase will help to account for the impressive capability of *F. succinogenes* to colonize and degrade forage cell walls (1, 14, 40).

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